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Antibodies against basic fibroblast growth factor inhibit the autocrine growth of pulmonary artery endothelial cells

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Anti-recombinant human basic fibroblast growth factor (rbFGF) antibodies efficiently inhibited the basal proliferation of bovine pulmonary artery endothelial (BAE) cells. The cell-free extract of BAE cells stimulated the proliferation of bovine capillary endothelial cells and this activity was completely abolished by the antibodies. Furthermore, on heparin HPLC the activity was eluted at exactly the same retention time as that for authentic pituitary bFGF. These observations directly indicate that the BAE cells produce bFGF that stimulates their own basal growth by binding to specific receptors expressed on the cell surface.

Fibroblast growth factor; Autocrine growth; Angiogenesis; (Endothelial cell)

1. INTRODUCTION

bFGF is a potent mitogen of vascular and capillary endothelial cells [1–3] and has been demonstrated to induce angiogenesis [2,4,5]. Vascular and capillary endothelial cells produce bFGF [6,7] and specific receptors are expressed on the cell surface [8–11]. These observations suggest that bFGF might be an autocrine growth factor of endothelial cells. However, there is no direct and clear evidence to support this hypothesis. It remains unsolved whether bFGF produced by the endothelial cells exerts its mitogenic effect via receptors expressed on the cell surface. To assess this possibility, we have studied the effect of anti-rbFGF antibodies on the growth of BAE cells and have found that the antibodies efficiently inhibit the growth of the cells. To the best of our knowledge, this is the first report directly revealing that the bFGF-dependent autocrine growth mechanism participates in the growth of vascular endothelial cells.

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Abbreviations: BAE, bovine pulmonary artery endothelial; BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; rbFGF, recombinant human basic fibroblast growth factor

2. MATERIALS AND METHODS

2.1. *Materials*

Homogeneous bovine pituitary bFGF was obtained as described previously [12]. Bovine brain acidic FGF was purchased from R & D Systems (USA) and rabbit normal IgG from Miles Laboratories (USA). Tissue culture multiwell plates (2 cm^2/well) were obtained from Flow Laboratories.

2.2. *Cell culture*

BAE cells were purchased from American Type Culture Collection (CCL-207). They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (MA Bioproducts, MD, USA), 20 units/ml penicillin G, and 100 μg/ml streptomycin. The cells were used between passage numbers 17 and 21. BCE cells were kindly provided by Dr J. Folkman and Ms C. Butterfield, Harvard Medical School. The cells were cultured in the same medium supplemented with rbFGF (2 ng/ml) in a gelatin-coated plastic flask under an atmosphere of 10% CO_2. The cells were used between passage numbers 9 and 11.

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2.3. *Preparation of cell-free extract*

Confluent BAE cells (CCL-207, 3×10^7 cells/750 cm^2) were detached using a cell scraper (C.A. Greiner und Söhne, FRG) and were washed twice with phosphate buffered saline (PBS). The washed cells were suspended in 0.6 ml PBS and were lysed by sonication (25 s at 0°C). The cell lysate was centrifuged at 12000 rpm in a microfuge for 5 min at 0°C to obtain 0.7 ml of cleared cell-free extract. It was stored in aliquots at −20°C until used.

2.4. *Fractionation of cell-free extract*

The cell-free extract (0.5 ml) was filtered with a 0.45 μm filter and applied to a heparin affinity HPLC column equilibrated at room temperature with 20 mM sodium phosphate buffer (pH 7.4) containing 0.4 M NaCl. The proteins were eluted with an NaCl gradient (0.4 M–1.7 M) at a flow rate of 1 ml/min. Fractions (1 ml) were collected and the mitogenic activity for BCE cells was measured.

2.5. *Preparation of rbFGF and anti-rbFGF antibodies*

Recombinant human bFGF was expressed in *E. coli* and purified to homogeneity as described [13] with a slight modification. Briefly, *E. coli* cells were disrupted by BEAD-BEATER (Biospec Products, USA), and the extract was applied to a CM-Toyopearl (Tosoh, Tokyo, Japan) column equilibrated with 50 mM sodium phosphate buffer (pH 6.0). Proteins were eluted with 50 mM sodium phosphate buffer (pH 6.0) containing 1 M NaCl. The active fractions were pooled and applied to a heparin affinity HPLC column (Shodex AF pack, Showadenko, Japan) and proteins were eluted with an NaCl gradient. The peak eluate consisted of highly purified rbFGF which gave a single band on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (fig.2, lane 3).

Rabbits were immunized twice at an interval of two weeks by multisite intradermal injections of 300 μg each of rbFGF emulsified with complete Freund's adjuvant. A week later, the rabbits were boosted with an intravenous injection of 300 μg rbFGF dissolved in saline. The titer of the antiserum after the booster injection was over 1×10^6 as determined in an ELISA. Whole IgG fractions were obtained from the antiserum by protein A-Sepharose affinity chromatography [14] and used throughout this work.

2.6. *Immunoblot analysis*

SDS-PAGE was performed as described [15] and immunoblotting was carried out essentially as described previously [16]. The nitrocellulose paper was incubated with anti-rbFGF antibodies and the antigen-antibody complex was visualized by successive incubation with peroxidase-conjugated anti-rabbit IgG mouse IgG (BioRad, CA, USA), 4-chloro-1-naphthol and H_2O_2.

3. RESULTS

Fig.1 shows that the growth of BAE (CCL-207) cells was stimulated by exogenous rbFGF. The cells were able to grow in the absence of exogenous rbFGF although the growth rate was somewhat smaller. To examine the participation of bFGF in

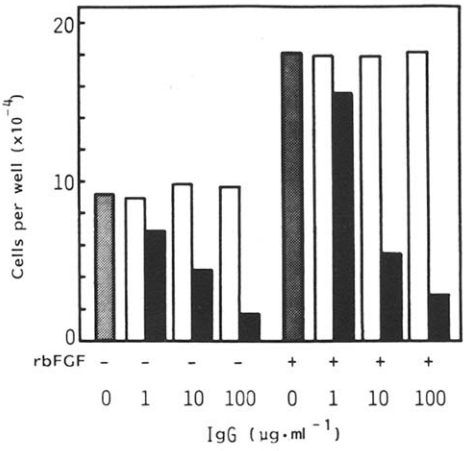

Fig.1. Effect of anti-bFGF antibodies on the proliferation of BAE cells. BAE (CCL-207) cells were plated at a density of 1×10^4 cells per well (2 cm^2) and cultured in the presence (+) or absence (−) of rbFGF (10 ng/ml). Anti-rbFGF antibodies (solid bars) or control IgG (open bars) were added at the beginning of culture. Dotted bars represent the growth for BAE cells in the absence of antibodies. After 3 days, the cells were trypsinized and counted in duplicate with a Coulter particle counter.

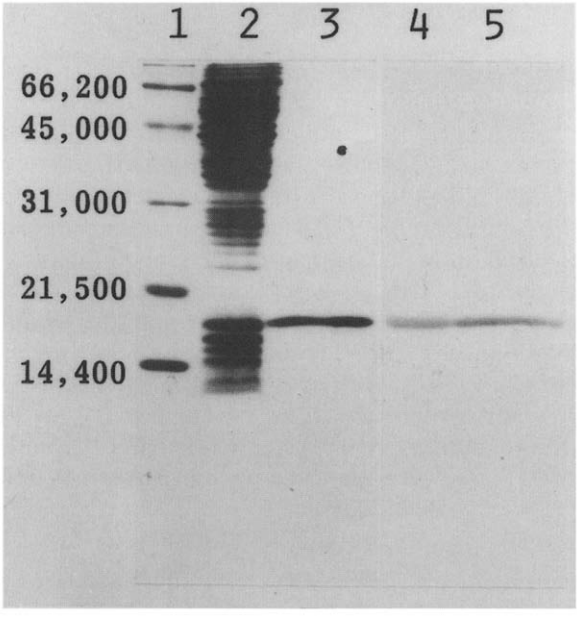

Fig.2. Immunoblot analysis with anti-rbFGF antibodies. Molecular mass markers (lane 1), purified rbFGF (lane 3, 2 μg; lane 5, 0.2 μg) and whole *E. coli* proteins (lanes 2 and 4, 50 μg) were subjected to SDS-PAGE. Lanes: 1–3, stained with Coomassie brilliant blue R-250; 4–5, the proteins were transferred to nitrocellulose paper and visualized using the anti-rbFGF antibodies as described in section 2.

the basal growth, we studied the effect of the anti-rbFGF antibodies. First, we checked the specificity of the antibodies by immunoblot analysis. As shown in fig.2, the antibodies reacted with rbFGF and showed no other cross-reacting bands in the *E. coli* lysate, indicating that the antibodies are specific to rbFGF. The antibodies were also confirmed to react with bFGF derived from bovine pituitary by immunoblot analysis (not shown). Since these antibodies were raised to rbFGF produced in *E. coli*, existence of antibodies against any other eucaryotic cell growth factors can be excluded.

The antibodies drastically inhibited the growth of BAE (CCL-207) cells in both the presence and absence of exogenous rbFGF, while normal IgG showed no effect (fig.1).

To confirm that the BAE cells used in this study produced bFGF as reported for other endothelial cells [6,7], a BAE cell-free extract was examined for its proliferative activity on BCE cells. As shown in fig.3, the extract induced the proliferation of the cells; 1 μl of the extract was as effective as about 2 ng of rbFGF. Coincidently, a large amount of bFGF (1.6 μg/ml extract) was detected by radioimmuno assay in the cell-free extract (Dr M. Yamaoka, personal communication). This activity, as well as the basal growth of BCE cells, was completely neutralized by the anti-bFGF antibodies. The cell-free extract was fractionated on a heparin HPLC column (fig.4). The mitogenic activity for BCE cells was eluted at the same position as bovine pituitary bFGF. No activity was detected at 0.6 M NaCl on the column, where bovine pituitary acidic FGF is eluted.

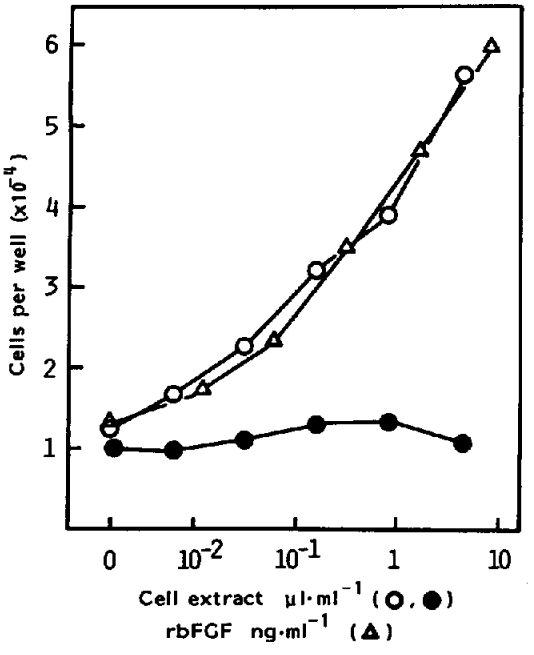

Fig.3. The effect of BAE cell-free extract on the proliferation of bovine capillary endothelial cells. BCE cells were plated at a density of 1×10^4 cells per 2 cm^2 well of gelatin-coated multiwell plate and cultured in the presence of the indicated concentrations of rbFGF (△), or BAE cell-free extract (○,●) with (●) or without (○) 13 μg/ml of anti-rbFGF antibodies. After 3 days, the cells were counted as described in fig.1.

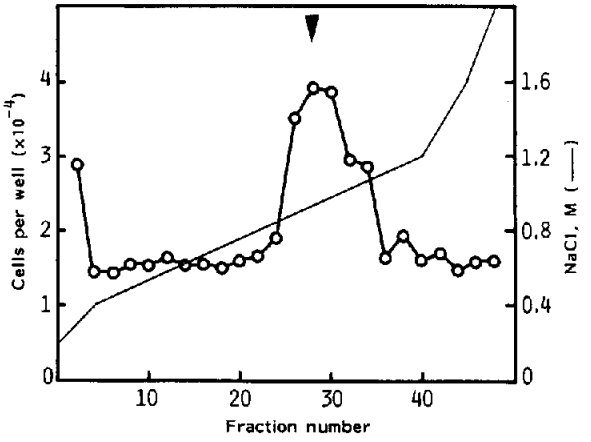

Fig.4. Fractionation of BAE cell-free extract on a heparin affinity HPLC. BAE cell-free extract was fractionated as described in section 2. 10 μl of each fraction was applied to BCE cell culture in 2 cm^2 well and assayed as described in fig.3. The elution position of bovine pituitary bFGF is indicated by an arrowhead.

4. DISCUSSION

The inhibitory effect of the anti-rbFGF antibodies on the BAE cells clearly indicates that the bFGF acts via specific receptors expressed on the cell surface. The BAE cells produce a large amount of mitogenic activity and store it intracellularly. From the reactivity with the specific anti-rbFGF antibodies (fig.3) and the chromatographic behavior on heparin HPLC (fig.4), it was confirmed that the activity was due to bFGF. Taken together, there is no doubt that BAE cells produce bFGF and that it induces the proliferation of BAE cells via an extracellular autocrine loop.

On the other hand, we were unable to detect bFGF activity in the conditioned medium of BAE cells by the mitogen assay (less than 0.04 ng/ml). Vlodavsky et al. [6] pointed out that bovine vascular aortic endothelial cells isolated from aortic arches synthesize and deposit the bFGF into the subendothelial extracellular matrix. It is likely that the bFGF produced by BAE cells is also deposited into the matrix and exerts the proliferative effect.

Schweigerer et al. [7] reported that bovine capillary endothelial cells also produce bFGF and suggested that bFGF exerts the effect via receptors on the cell surface. However, their rabbit antibodies showed only very weak inhibition of the basal growth of capillary endothelial cells. Although the low efficiency may have resulted from masking of the epitopes of endogenous bFGF by the extracellular matrix [7], it did not provide direct evidence for the autocrine growth stimulation model.

Consistent with our results, Sasada et al. [17] reported that the morphologic transformation induced by the expression of human bFGF cDNA in mouse BALB/c3T3 was reversed by adding our anti-rbFGF antibodies. It was also concluded that bFGF is produced and stored in the transformed mouse fibroblast cells and that it exerts its mitogenic effect via receptors on the cell surface.

bFGF has no typical signal sequence [18,19] suggesting that it may be transported to the outside of the cell via a different pathway from that of normal secretory proteins. The transport and secretion mechanisms are to be elucidated.

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